Connexin 32 increases the proliferative response of Schwann cells to neuregulin-1 (Nrg1)

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Connexin 32 (Cx32), a gap junction protein, is found within the para-nodal region and Schmidt-Lanterman incisures of myelinating Schwann cells (SCs). In developing and regenerating peripheral nerves, pro-myelinating SCs express Cx32 mRNA and protein in conjunction with the expression of myelin specific genes. Neuregulin-1 (Nrg1), a member of the neuregulin family of growth factors, controls SC proliferation and differentiation depending on the cellular environment and the particular stage of SC maturation. Primary cultures of purified SCs from newborn mouse sciatic nerve were used to characterize both the role of Nrg1 in the expression of Cx32 and, conversely, the role of Cx32 in SC responsiveness to Nrg1. Glial growth factor 2, an isoform of Nrg1, up-regulated Cx32 in both proliferating and non-proliferating SCs. However, SCs from Cx32-KO mice exhibited a significantly smaller mitogenic response to glial growth factor 2. Electrical coupling between Cx32-KO SCs did not differ from that between WT SCs, indicating the presence of other connexins. These results suggest a link between Cx32 expression and Nrg1 regulation of SC proliferation that does not involve Cx32-mediated intercellular communication.

Cx32 knockout | glial growth factor 2 | myelin | cell cycle | CMTX

In myelinating Schwann cells (SCs) of peripheral nerve, the gap junction protein connexin 32 (Cx32) is found within the non-compact myelin of the paranodal region and Schmidt-Lantermann incisures (1). Cx32 gap junctions between successive cytoplasmic loops shorten the radial diffusion pathway for ions and other small signaling molecules between the SC nucleus and adaxonal cytoplasm at least 300 times (2). Loss of Cx32-mediated coupling is thought to be the pathogenic mechanism in X-linked Charcot-Marie-Tooth disease (CMTX), an inherited peripheral neuropathy associated with mutations in the human gene for Cx32, *GJB1* (3). Supporting this idea, a mouse with targeted ablation of Cx32 develops histopathology resembling that seen in humans with CMTX (4).

SC expression of Cx32 is not limited to the mature myelinating phenotype. Cx32 mRNA and protein are co-regulated with SC expression of myelin-specific genes during development and in regenerating peripheral nerves (1, 5, 6). In mouse xenograft experiments, human nerve segments containing SCs with a predicted loss-of-function Cx32 mutation (Val181Ala) were severely impaired in their ability to support the earliest stages of regeneration of myelinated fibers, whereas nerve segments from a patient with a less severe mutation (Glu102Gly) and those from a normal subject supported normal early regeneration (7). The abnormalities associated with the V181A mutation occur at a time when no myelin is present, further supporting a function for Cx32 in non-myelinating SCs.

Neuregulins, including Nrg1, Nrg2, and Nrg3, are a family of growth factors that influence the survival, differentiation, and myelinating potential of peripheral and central nervous system glial cells (reviewed in ref. 8). ErbB2/ErbB3 heterodimers are neuregulin receptors and are required for SC development (9). Nrg1 controls SC phenotype depending on cellular environment and the specific stage of SC differentiation or maturation (10). Neuronal/axonal sources of neuregulin provide trophic support for immature SCs, promote myelin formation, and regulate myelin thickness during development (11). In mature peripheral nerve, Nrg1 participates in the regeneration and re-myelination of injured myelinated fibers, processes that involve SC de-differentiation, proliferation, and re-differentiation to a myelinating phenotype (12).

Glial growth factor 2 (GGF2) is derived from a splice variant of the Nrg1 gene transcript. Isoforms of Nrg1, such as GGF2 and Nrg1-III, promote survival of cultures of SC progenitors (13). GGF2 rescues cultured SCs after serum withdrawal and up-regulates the expression of pro-myelinating SC markers (14, 15). Furthermore, addition of GGF2 to myelinating co-cultures of SCs and sensory neurons causes demyelination and stimulates SC de-differentiation and proliferation (12).

Primary cultures of purified SCs from newborn mouse sciatic nerve were used to characterize the role of GGF2 in SC expression of Cx32 and to determine whether expression of Cx32 alters SC responsiveness to it. We found that Cx32 expression was up-regulated by GGF2 and that GGF2 stimulated Cx32 expression in proliferating SCs as well as in non-proliferating SCs. These results suggest that Cx32 and GGF2 may interact through an amplification mechanism such that GGF2 increases expression of Cx32 in proliferating cells and Cx32 expressing cells are more sensitive to the proliferative effects of GGF2.

Results

SCs in culture show a pattern of gene expression typical of SCs after nerve injury (16–18). We used cultures of primary SCs to characterize the function of Cx32 in non-myelinating SCs. Cultures of purified SCs were established from newborn (postnatal day 3–5) mouse sciatic nerves using standard techniques with some modifications (16, 19) (see *Methods*).

GGF2 Increases Proliferation More in WT than in Cx32-KO SCs. To test the hypothesis that expression of Cx32 plays a role in the proliferative SC response to neuregulin, we examined proliferation in GGF2-treated cultured SCs from age-matched neonatal WT and Cx32-KO (32KO) mice. WT and 32KO SCs at 2 and 6 days in vitro (DIV) were treated overnight with BrdU (10 μM) in the presence or absence of GGF2 (5 ng/mL) and processed for incorporation of BrdU [Fig. 1 *A* and *B* and supporting information (SI) Fig. S1]. After 2 DIV, overnight treatment with GGF2 led to a significant increase in the percentage of BrdU-labeled WT SCs but not of 32KO SCs. Both GGF2-treated and control WT SCs showed significantly higher BrdU-labeling than the corresponding 32KO cultures. At 6 DIV, overnight GGF2 treatment increased BrdU-labeling in WT cultures but not in

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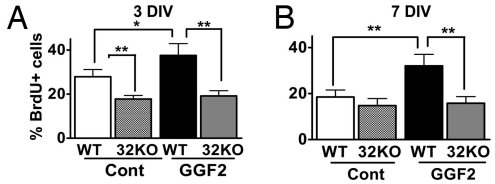

Fig. 1. GGF2 increases proliferation in WT SCs compared with 32KO. After 2 or 6 DIV, WT and 32KO cultured SCs were incubated with BrdU (10 μM) overnight in the presence or absence of GGF2 (5 ng/mL) and fixed the next day at 3 DIV (*A*) or 7 DIV (*B*). (No. of images, 3 DIV, WT control, $n = 26$; WT GGF2, $n = 16$; 32KO control, $n = 27$; 32KO GGF2, $n = 17$; 7 DIV: WT control, $n = 25$; WT GGF2, $n = 18$; 32KO control, $n = 32$; 32KO GGF2, $n = 21$; **, $P < 0.01$, *, $P < 0.05$.)

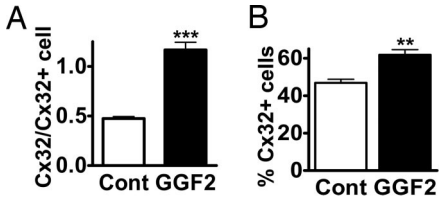

Fig. 2. GGF2 increased both the percentage of SCs that were Cx32-positive and the level of Cx32 in Cx32-positive cells. SCs after 6 DIV were cultured overnight in GGF2 (5 ng/mL) or control medium, immuno-labeled with affinity-purified polyclonal antibody to Cx32 (EL1), and counterstained with DAPI. Fluorescence signals were quantified as described in *Methods*. (*A*) Mean Cx32 fluorescence per Cx32-positive cell (in arbitrary units) was increased to 2.46 times control value by GGF2 treatment. (No. of cells: control, $n = 1{,}972$; GGF2, $n = 2{,}082$; **, $P < 0.01$.) (*B*) Percentage of Cx32-positive cells was determined for each image from the total number of nuclei and total number of Cx32-labeled (Cx32+) SCs. Percentage of Cx32-positive cells was increased to 1.25 times control by GGF2 treatment. (No. of images, $n = 65$; ***, $P < 0.001$.)

Cx32 KO cultures; and no significant differences were noted in BrdU labeling between control WT and 32KO SCs. To characterize the kinetics of 32KO SC proliferation and response to GGF2, Alamar Blue precursor was applied to control and GGF2-treated WT and 32KO cultures for up to 2 d after 6 DIV (Fig. S2). Alamar Blue fluorescence is a measure of total cytoplasmic reducing capacity of metabolically active cells; however, over time the availability of reaction product may decline, affecting the linearity of the measurements (20). At 7 DIV or 24 h after treatment, Alamar Blue signal was increased significantly in GGF2-treated WT SCs versus WT controls, in correlation with the increased proliferation at 7 DIV assayed by BrdU immunolabeling. By contrast, increases in Alamar Blue signal in response to GGF2 did not become significant in 32KO SCs until after 48 h of treatment (8 DIV). Thus, the two measures of SC proliferation/metabolic activity were in qualitative agreement and demonstrated differences between WT and 32KO SCs in their responses to GGF2. Although decreased SC survival in 32KO cultures cannot be ruled out, visual inspection of WT and 32KO cultures at each time point did not indicate any alterations in cell morphology, nuclear fragmentation, cytoplasmic blebbing, increase in cellular debris, or any other sign of apoptotic or necrotic cell death. These findings are consistent with the hypothesis that loss of the Cx32 gene leads to attenuated mitogenic potential and a reduced proliferative response to GGF2, supporting a link between expression of Cx32 and Nrg1-mediated control of SC proliferation.

GGF2 Increases Cx32 Expression in Cultured SCs. The observation that SCs from 32KO mice show a reduced proliferative response to GGF2 raised the possibility that the GGF2 response may involve modulation of the expression of Cx32 in non-myelinating WT SCs. We used cultured SCs from neonatal WT mice to examine GGF2 modulation of Cx32. After 8 DIV, SC cultures were treated with 5 ng/mL GGF2, a concentration that gave a readily detectable response (Fig. S3).

Treatment with GGF2 induced an increase in Cx32 immunofluorescence in cultured WT SCs (Fig. 2 and Fig. S4) as a result of increase in both the amount of Cx32 per Cx32-positive cell (to 2.46 times control; Fig. 2*A*) and the percentage of SCs labeled for Cx32 (to 1.25 times control; Fig. 2*B*). Thus, the overall increase was to approximately 3 times control.

The affinity purified polyclonal anti-Cx32 antibody used in these studies (EL1, a gift from E. Hertzberg; see *Methods*) gave little or no staining of 32KO SCs, confirming its specificity (Fig. S5). The specificity of 7c6.7c, a mouse monoclonal anti-Cx32 (a gift from E. Hertzberg, NY), was confirmed by Western blot analysis using WT and 32KO tissues and immunoprecipitation from lysates of cultured SCs (Fig. S6).

Earlier studies characterizing the proliferative and differentiating effects of GGF2 used rat and mouse SCs grown and maintained in serum-containing media (21). We found that GGF2 treatment of SC cultures maintained in serum-free B27NBM$_A$ also elicited up-regulation of Nrg1 receptors ErbB2 and ErbB3 (Fig. 3) (21), increases that correlate positively with GGF2-stimulated increases in Cx32 as described earlier.

Proliferation and Expression of Cx32 in Cultured SCs. As described earlier, exposure to GGF2 increased WT SC labeling for BrdU by approximately 70% at 7 DIV, but did not alter BrdU labeling of SCs from 32KO mice (Fig. 1*B*). We therefore examined the relation between GGF2-induced proliferation and Cx32 expression. After 6 DIV, WT SCs were treated overnight with BrdU with or without GGF2 and double-labeled for Cx32 and BrdU. GGF2 increased mean levels of Cx32 in both BrdU-positive (i.e., proliferating) and BrdU-negative (i.e., non-proliferating) cells, but more so in BrdU-positive cells (Fig. 4). In addition, GGF2 treatment increased the percentage of total cells that were co-labeled for BrdU and Cx32 from 3.6% (30 of 838) to 6.5% (55 of 842; $P = 0.0073$ Fisher exact test). These data are consistent with a relation between the mitogenic and Cx32-regulatory effects of Nrg1.

Forskolin Increases Cx32 Expression in Cultured SCs. GGF2 treatment of cultured SCs elevates intracellular levels of cAMP, and this response is associated with the mitogenic effects of GGF2 in either the presence or absence of serum (22). Treatment with forskolin or cAMP analogs alone is not sufficient to promote proliferation in cultured SCs (23). However, forskolin does promote expression of pro-myelinating SC markers P0, MBP, and MAG, genes whose SC expression correlates with that of Cx32 (24). As observed with GGF2, Western blot analysis and

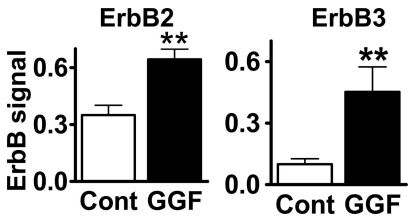

Fig. 3. GGF2 increases ErbB2 and ErbB3 expression in SCs. Dissociated WT SCs were seeded into 96-well plates. At 6 DIV, SCs were treated overnight with GGF2 (5 ng/mL) or buffer. The cells were fixed and immuno-labeled using polyclonal antibodies to either ErbB2 or ErbB3 and counterstained with Hoechst. ErbB2, ErbB3, and Hoechst fluorescence signals were measured in a Flexstation II microplate reader (Molecular Devices). Total integrated ErbB2 or ErbB3 fluorescence signal was normalized to the corresponding Hoechst signal. (No. of wells per group: $n = 8$, **, $P < 0.01$.)

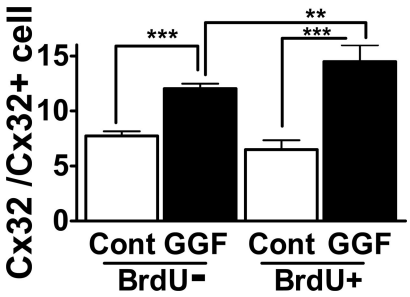

Fig. 4. GGF2 increases Cx32 expression in sub-populations of proliferating and non-proliferating cultured SCs. Dissociated WT SCs were seeded onto coverslips and, after 6 DIV, incubated overnight with BrdU (10 μM) in the presence or absence of GGF2 (5 ng/mL). Cells were double-labeled with anti-Cx32 (EL1) and anti-BrdU and counterstained with DAPI. Cells were divided into BrdU-positive and BrdU-negative groups, and mean integrated Cx32 fluorescence per cell (in arbitrary units, see *Methods*) was determined for each group in control and GGF2-treated cultures. The relatively small population of BrdU-positive SCs showed significantly higher Cx32 signal per cell than the subpopulation of BrdU-negative/Cx32-positive SCs. (No. of cells: control BrdU-negative, $n = 358$; GGF2 BrdU-negative, $n = 370$; control BrdU-positive, $n = 30$; GGF BrdU-positive, $n = 55$; ***, $P < 0.001$, **, $P < 0.01$.)

immunostaining demonstrated an increase in levels of Cx32 by Western analysis with increasing concentrations of forskolin (Fig. S7*A*) along with significant increases in Cx32 per Cx32-positive cell (Fig. S7*B*) and the percentage of cells that were Cx32-positive (Fig. S7*C*).

Nrg1 signaling involves protein kinase A (PKA)/cAMP-mediated transduction pathways, among other well described cell signaling mechanisms (25). To determine whether blockade of PKA signaling pathways would suppress the effects of GGF2 on Cx32 expression, WT SCs were pretreated for 30 min with PKA inhibitor peptide (PKAInh, a myristoylated cell-permeable specific PKA inhibitor peptide) or H8 (a less selective PKA inhibitor) followed by overnight treatment with inhibitor and GGF2 (5 ng/mL). PKAInh and H8 suppressed the effects of GGF2 on SC Cx32 (Fig. 5), indicating that PKA-signaling mediates GGF2 control of SC Cx32. Treatment with PKAInh blocked the effects of forskolin on Cx32 abundance, confirming the efficacy of the blocker and further demonstrating that Cx32 expression can be regulated directly through cAMP signaling pathways (Fig. S8). Whereas GGF2 and forskolin each stimulate Cx32 via cAMP/PKA-mediated transduction pathways, other Nrg1 subtypes might regulate Cx32 expression through additional ErbB2/ErbB3 signaling pathways.

GGF2 Regulation of Junctional Coupling. Cx32-mediated coupling in proliferating SCs would be consistent with the concept that

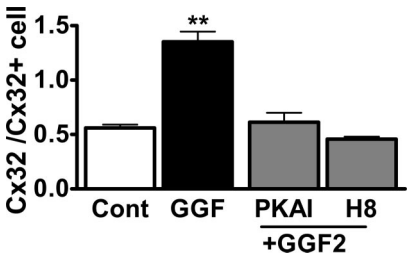

Fig. 5. Inhibition of PKA blocks the effects of GGF2 in increasing Cx32 expression in Cx32-positive SCs. Cultured WT SCs at 7 DIV were pretreated 30 min with PKAInh (350 nM; $K_i = 36$ nM) or H8 (5 μM; $K_i = 1.2$ μM) or buffer before overnight treatment with inhibitor plus GGF2 (5 ng/mL). Coverslips were immuno-labeled with anti-Cx32 (EL1) and counterstained with DAPI. (No. of cells: control, $n = 1,242$; GGF2, $n = 1,712$; GGF2 + PKAInh, $n = 1,314$; GGF2 + H8, $n = 1,392$; ***, $P < 0.001$.)

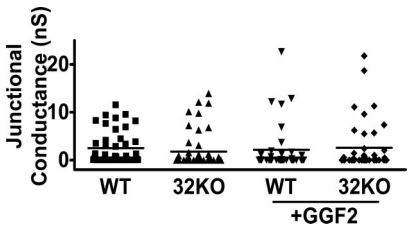

Fig. 6. Gap g_j does not differ significantly among WT and 32KO SCs with and without GGF2 treatment. Cells were maintained in $B27NBM_A$ for 1 to 9 DIV, treated overnight with GGF2 (5 ng/mL), and examined using dual whole-cell patch clamping. Untreated WT and 32KO sister cultures were examined in parallel. Cell pairs from all 4 groups show similar distributions of junctional coupling. Horizontal bars indicate mean g_j.

Cx32-mediated coupling is required for normal function of proliferating SCs, whereas Cx32 protein expression without coupling would suggest a non-junctional function in these cells. To examine this question further, junctional coupling was measured between isolated pairs of WT and 32KO SCs treated for 24 h with GGF2 or control medium after 1 to 9 DIV (Fig. 6). Surprisingly, junctional conductance (g_j) between unstimulated WT and 32KO SC pairs did not differ significantly (2.5 ± 0.5 nS, $n = 43$ for WT; 1.78 ± 0.5 nS, $n = 53$ for 32KO). Moreover, GGF2 did not significantly increase the mean g_j for either WT or 32KO cell pairs (2.2 ±.1 nS, $n = 37$ for WT; 2.6 ± 0.8 nS, $n = 42$ for 32KO). As with measurements of magnitude of conductance, evaluation of the trans-junctional voltage dependence and unitary conductances in the 4 groups of cell pairs showed no differences between WT and 32KO or between GGF2-treated and untreated cells. A range of single channel conductances was observed, not restricted to the ≈65 pS expected for Cx32. These data suggest that other connexins are likely to underlie the coupling in SCs (see *Discussion*).

Discussion. Here we show that SC expression of Cx32 is up-regulated by treatment with the Nrg1 isoform GGF2; however, there is no corresponding increase in electrical coupling between SCs following exposure to GGF2. Remarkably, coupling conductance was not significantly different in any of the 4 groups: WT and 32KO with and without GGF2 treatment. In untreated controls, the percentage of SCs expressing Cx32 is similar in proliferating and non-proliferating subpopulations (BrdU-positive and BrdU-negative); however, treatment with GGF2 increases the level of expression more in proliferating than in non-proliferating SCs. Finally, SCs from 32KO mice show a striking absence of mitogenic response to GGF2 at 3 DIV and at 7 DIV, in contrast to the significant GGF2-stimulated increase in proliferation observed in WT cultures. These findings strongly suggest a role for Cx32 in Nrg1-mediated proliferation, but not one mediated by electrical coupling.

The experiments presented differ from many prior studies in that the SC cultures were grown and maintained in serum-free defined medium (after an initial period ≤24 h in bovine growth serum). Serum-free defined medium was used to preclude any effects from factors present in animal sera. For example, FBS is mitogenic for cultured SCs and alters responses to trophic factors (26, 27).

The particular SC response to neuregulin involves a complex interplay between the differentiation state of the SC, the concentration and duration of Nrg1 exposure, and the resulting activation of multiple signaling pathways. Ligand activation of the ErbB2/ErbB3 receptor complex triggers elements in the PKA/cAMP, PI3K/Akt, and MAPK/ERK signaling pathways in proliferating and myelinating SCs (25, 28). Inhibition of PI3K impedes SC proliferation, interferes with Nrg1 rescue of serum-

deprived SCs, and inhibits the formation of myelin in SC-neuron co-cultures (24, 29). Nrg1 treatment of myelinated co-cultures, however, causes profound demyelination and SC dedifferentiation and proliferation through phosphorylation of PI3K and MEK (30).

Forskolin and cAMP analogues mimic some of the effects of axonal contact on SCs and induce SC differentiation toward a myelinating phenotype, including up-regulation of myelin proteins P0, MAG, and MBP, and down-regulation of non-myelinating markers GFAP and N-CAM (23, 31). We found that treatment with forskolin, which increases intracellular levels of cAMP, also enhances SC expression of Cx32. Increases in intracellular cAMP alone are not sufficient to stimulate SC proliferation, as neither forskolin nor cAMP is mitogenic for SCs grown without serum or other trophic agents (17). However, forskolin significantly enhances the SC mitogenic responses to several growth factors, including Nrg1, PDGF, and insulin-like growth factor-1 (17, 23). Forskolin also augments Nrg1-dependent SC proliferation by accelerating G1-to-S-phase cell cycle progression through stimulation of cyclin D, prolonged activation of ERK signaling, and increased PI3K/Akt activity (23, 32). The effects of forskolin and Nrg1 involve common intracellular signaling cascades, including the MAPK and PI3K pathways. The balance of activities between these pathways appears crucial in modulating SC function.

Cx32 expression was significantly elevated in proliferating, BrdU-positive WT SCs following treatment with GGF2. In addition, inhibition of PKA suppressed the effects of both GGF2 and forskolin on Cx32. Inhibition of PKA hinders the proliferative effects of forskolin and can interfere with SC myelin formation in neuron/SC co-cultures (22, 33). Monje and colleagues (34) recently reported that PKA activity has a synergistic gating action by enhancing GGF-mediated ErbB2/ErbB3 activation leading to SC proliferation. Although cAMP-mediated pathways appear to be involved in regulating both Cx32 and SC number and differentiation, it remains unclear whether these effects are directly associated with the same ErbB2/ErbB3 signaling pathways. We have shown that either forskolin or GGF2 can increase Cx32 expression, and that these agents exert their effects on Cx32 expression through PKA-mediated cell signaling pathways.

Nicholson and colleagues (35) described provocative findings suggesting WT and 32KO cultured SCs differ in their predominant phenotypic stage of development; specifically that 32KO SCs expressed high levels of GFAP, an early SC marker and indicator of non-myelinating phenotype. They also found that WT and 32KO SCs responded to treatment with GGF2 with increased proliferation at 8 to 12 DIV. We observed similar rates of BrdU incorporation in WT and 32KO SCs at 7 DIV. However, we found that treatment with GGF2 failed to stimulate proliferation in 32KO SCs. Closer examination suggests several differences. First, Nicholson and co-workers established cultured SCs in high serum (15%), which can lead to the generation of sub-populations of spontaneously immortalized SCs and a significant enrichment of de-differentiated SCs (55). Second, the cultures were maintained in 2% serum, which contains growth factors that might interact with GGF2. Third, these SCs were isolated from Cx32-null mice with a mixed strain background. We have found distinct differences in rates of proliferation in SCs cultured from different mouse strains. For this reason, we used extensively back-crossed 32KO-C57BL/6 mice (provided by S.A.L. Bennett, Ottawa, ON, Canada) to eliminate possible strain effects. Despite these experimental differences, the observation that absence of the Cx32 gene leads to an increase in SC de-differentiation and deficit in responsiveness to differentiating signals is concordant with our observation of reduced mitogenic response to GGF2 in 32KO SCs.

Patch-clamp experiments showed no significant differences between the levels of junctional coupling in SC pairs from WT or 32KO mice, either with GGF2 stimulation or without. This implies that the absence of Cx32 has no impact on junctional coupling under these conditions. In addition, it supports the notion that any effect of Cx32 is likely to be independent of gap junction formation. In agreement with our results for GGF2, Chandross and colleagues showed no significant change in electrical coupling in response to treatment with neu differentiation factor-β, a form of GGF (36). Earlier reports of SC coupling differ from those reported here in the magnitude of coupling (36, 37). However, species differences (e.g., rat vs. mouse), culture conditions (e.g., serum-containing vs. defined medium), as well as time in culture (e.g., up to 6 weeks vs. 2–10 d) may account for these divergent findings.

Taken together, these findings suggest that Cx32 is entirely non-junctional in non-myelinating SCs and that presence of cytoplasmic Cx32 alters SC responses to the differentiating and proliferative effects of GGF2. Recent studies present intriguing associations for expression of non-junctional Cx32 and the control of proliferative capacity in some cell types. For example, phenobarbital promotes liver tumors rats and causes increased cytoplasmic Cx32 staining in centrolobular liver cells (38). Increases in cytoplasmic Cx32 protein also favor hepatocellular carcinoma progression once the cells have acquired a malignant phenotype, further supporting an association between non-junctional Cx32 expression and activation of cell cycle machinery (39, 40).

The influence of Cx32 expression on cell proliferation appears to be cell type-specific. Loss of Cx32 expression appears to remove a level of regulatory control of cell cycle for tumor cells or pluripotent progenitor cells (41–43). Melanson-Drapeau and colleagues (44) have observed an increase in oligodendrocyte-progenitor-cell proliferation in the dentate gyrus of Cx32-deficient mice. Cx32KO mice develop hepatic tumors at a rate much greater than WT. However, transgenic mice expressing a dominant-negative form of Cx32 (Val139Ala) show delayed liver regeneration and greater susceptibility to diethylnitrosamine-induced liver tumors. Exogenous expression of Cx32 suppresses proliferation in several cell lines (45, 46). Finally, our findings suggest that Cx32 acts to promote proliferation in non-myelinating SCs. Thus, expression of Cx32 can coordinate cell growth both positively and negatively and may be essential for homeostasis in certain cell types. Although Cx32 may be required for optimal growth in some cell types, it does not act exclusively as a suppressor or enhancer of growth, an idea first advanced by Omori and co-workers (40).

Non-junctional actions of connexins include binding to other proteins and possibly acting as trafficking or scaffolding proteins (47–49). In addition, connexins can form un-apposed hemichannels in non-junctional membranes and release such potential signaling molecules as ATP, IP3, NAD^+, and glutamate (48, 50). CMTX mutations may act through increased opening as hemi-channels (51).

The proposal that proliferation and Cx32 expression are regulated in parallel by GGF2 fits with the accepted role of Nrg1 in directing SC phenotype expression during developmental and regenerative processes (52). In the mouse, Wallerian degeneration induces SC proliferation and increases expression of Nrg1 and ErbB receptors, with a maximal response at approximately 3 to 5 days after injury (53). GGF and other Nrg1 isoforms, acting through neuronal (i.e., paracrine) and SC-mediated (i.e., autocrine) pathways, can stimulate SC survival and proliferation in response to denervation as suggested by previous molecular studies (54).

We found that Cx32 is positively regulated by GGF2 in a population of proliferating and non-proliferating SCs. Moreover, in the absence of Cx32, SCs have a reduced mitotic response to GGF2 by 7 DIV. These results suggest a possible link

between Cx32 expression and neuregulin regulation of SC proliferation. Our finding that GGF2 did not alter the expression of electrical coupling in proliferating SCs suggests that coupling across cells is not a mechanism for amplification of NRG-1 signaling. Unpublished findings from real-time PCR experiments suggest that other connexins are expressed in SCs and that some of these are also regulated by GGF2.* Further studies will be required to determine which connexins contribute to SC gap junction coupling.

Methods

Animals. Timed pregnant female C57BL/6 mice were obtained from Charles River Laboratories. Sciatic nerves were obtained from postnatal P3–5 day WT C57BL/6 mice. A colony of extensively backcrossed 32KO/C57BL6 mice (32KO) was bred in-house (founders provided by S.A.L. Bennett, Ottawa, ON, Canada). All animals were provided food and water ad libitum and housed according to the Guide for the Care and Use of Laboratory Animals.

SC Cultures. SC cultures were established using a combination of standard methods (19, 32), with some modifications as described in *SI Methods*. The dissociated SCs were transferred to B27NBM$_A$ [Neural Basal A Medium (Invitrogen); 0.5 mM GlutaMax (Invitrogen); 10 mM Hepes, pH 7.4; B27 supplement (Invitrogen); and Primocin (InvivoGen)] supplemented with 10% bovine growth serum (HyClone) and seeded onto polyD-lysine-coated dishes at 0.23 sciatic nerve equivalents/cm^2 supplemented with GGF2 (5 ng/mL' gift from Cambridge Neurosciences/Acorda) and forskolin (10 μM; Sigma). Eighteen to 24 h after plating, cultures were switched to serum-free B27NBM$_A$ supplemented with GGF2 and forskolin. GGF2 and forskolin supplements were withdrawn when the cultures reached approximately 90% confluence at 4 to 5 DIV and maintained in B27NBM$_A$ alone thereafter.GGF2 and forskolin supplementation was always discontinued at least 24 h before any experimental treatment.

Immunocytochemistry. Cultures were fixed at room temperature with Histochoice (Amresco), permeabilized, and blocked with ICC block. Coverslips were processed for immunofluorescence as described in *SI Text*. Digital images were collected and analyzed using Metamorph 7 image analysis software (Molecular Devices) as described in *SI Text*.

Proliferation and Survival Assays. ***BrdU Incorporation.*** Cultures were treated with BrdU (10 μM; Molecular Probes) overnight in the presence or absence of GGF2, fixed, and processed as described in *SI Methods*.
Alamar Blue. Cultures were seeded onto black-wall, clear-bottom 96-well plates (Costar/Corning). Alamar Blue precursor (Invitrogen/Biosource) was added to a final concentration of 10% (vol/vol), along with GGF2 and buffer treatments. Alamar Blue fluorescence was read with a Flexstation II microplate reader (Molecular Devices).

Electrophysiology. Dual whole-cell patch clamping was performed as previously described on SC pairs 2 to 10 d after initial plating (7). Recording solutions were as follows: pipette solution, 145 mM CsCl, 5 mM EGTA, 0.5 mM $CaCl_2$, 10.0 mM Hepes, pH 7.2; bath solution, 150 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM dextrose, 2 mM pyruvate, 10 mM Hepes, pH 7.4. Bars in Fig. 6 represent mean g_j.

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1. Scherer SS, *et al.* (1995) Connexin32 is a myelin-related protein in the PNS and CNS. *J Neurosci* 15:8281–8294.
2. Abrams CK, Bennett MVL (2000) Hereditary human diseases caused by connexin mutations. *Gap junctions- Molecular Basis of Cell Communication in Health and Disease*, ed Peracchia C (Academic, New York), pp 423–424.
3. Abrams CK, Oh S, Ri Y, Bargiello TA (2000) Mutations in connexin 32: the molecular and biophysical bases for the x-linked form of Charcot-Marie-Tooth disease. *Brain Res Brain Res Rev* 32:203–214.
4. Scherer SS, *et al.* (1998) Connexin32-null mice develop demyelinating peripheral neuropathy. *Glia* 24:8–20.
5. Smith-Slatas C, Barbarese E (2000) Myelin basic protein gene dosage effects in the PNS. *Mol Cell Neurosci* 15:343–354.
6. Sohl G, *et al.* (1996) A second alternative transcript of the gap junction gene connexin32 is expressed in murine Schwann cells and modulated in injured sciatic nerve. *Eur* J Cell Biol 69:267–275.
7. Abrams CK, *et al.* (2003) Pathogenesis of x-linked Charcot-Marie-Tooth disease: Differential effects of two mutations in connexin 32. *J Neurosci* 23:10548–10558.
8. Esper RM, Pankonin MS, Loeb JA (2006) Neuregulins: Versatile growth and differentiation factors in nervous system development and human disease. *Brain Res Rev* 51:161–175.
9. Citri A, Skaria KB, Yarden Y (2003) The deaf and the dumb: the biology of ErbB-2 and ErbB-3. *Exp Cell Res* 284:54–65.
10. Lemke G (2006) Neuregulin-1 and myelination. *Sci STKE* 2006:pe11.
11. Michailov GV, *et al.* (2004) Axonal neuregulin-1 regulates myelin sheath thickness. *Science* 304:700–703.
12. Zanazzi G, *et al.* (2001) Glial growth factor/neuregulin inhibits Schwann cell myelination and induces demyelination. *J Cell Biol* 152:1289–1299.
13. Leimeroth R, *et al.* (2002) Membrane-bound neuregulin1 type III actively promotes Schwann cell differentiation of multipotent progenitor cells. *Dev Biol* 246:245–258.
14. Chen S, *et al.* (2006) Neuregulin 1-ErbB signaling is necessary for normal myelination and sensory function. *J Neurosci* 26:3079–3086.
15. Cheng L, Esch FS, Marchionni MA, Mudge AW (1998) Control of Schwann cell survival and proliferation: Autocrine factors and neuregulins. *Mol Cell Neurosci* 12:141–156.
16. Cheng L, Mudge AW (1996) Cultured Schwann cells constitutively express the myelin protein P0. *Neuron* 16:309–319.
17. Morgan L, Jessen KR, Mirsky R (1991) The effects of cAMP on differentiation of cultured Schwann cells: Progression from an early phenotype (04$^+$) to a myelin phenotype (p0$^+$, gfap$^-$, n-cam$^-$, ngf-receptor$^-$) depends on growth inhibition. *J Cell Biol* 112:457–467.
18. Wu R, Jurek M, Sundarababu S, Weinstein DE (2001) The POU gene Brn-5 is induced by neuregulin and is restricted to myelinating Schwann cells. *Mol Cell Neurosci* 17:683–695.
19. Brockes JP, Fryxell KJ, Lemke GE (1981) Studies on cultured Schwann cells: the induction of myelin synthesis, and the control of their proliferation by a new growth factor. *J Exp Biol* 95:215–230.
20. Nikolaychik VV, Samet MM, Lelkes PI (1996) A new method for continual quantitation of viable cells on endothelialized polyurethanes. *J Biomater Sci Polym Ed* 7:881–891.
21. Garratt AN, Britsch S, Birchmeier C (2000) Neuregulin, a factor with many functions in the life of a Schwann cell. *BioEssays* 22:987–996.
22. Kim HA, DeClue JE, Ratner N (1997) cAMP-dependent protein kinase A is required for Schwann cell growth: Interactions between the camp and neuregulin/tyrosine kinase pathways. *J Neurosci Res* 49:236–247.
23. Monje PV, Bartlett Bunge M, Wood PM (2006) Cyclic amp synergistically enhances neuregulin-dependent Erk and Akt activation and cell cycle progression in Schwann cells. *Glia* 53:649–659.
24. Ogata T, *et al.* (2004) Opposing extracellular signal-regulated kinase and Akt pathways control Schwann cell myelination. *J Neurosci* 24:6724–6732.
25. Ogata T, Yamamoto S, Nakamura K, Tanaka S (2006) Signaling axis in Schwann cell proliferation and differentiation. *Mol Neurobiol* 33:51–62.
26. Brockes JP, Raff MC (1979) Studies on cultured rat Schwann cells. II. Comparison with a rat Schwann cell line. *In Vitro* 15:772–778.
27. Morgan L, Jessen KR, Mirsky R (1994) Negative regulation of the P0 gene in Schwann cells: Suppression of P0 mRNA and protein induction in cultured Schwann cells by FGF2 and TGF beta 1, TGF beta 2 and TGF beta 3. *Development* 120:1399–1409.
28. Maurel P, Salzer JL (2000) Axonal regulation of Schwann cell proliferation and survival and the initial events of myelination requires PI3-kinase activity. *J Neurosi* 20:4635–4645.
29. Li Y, Tennekoon GI, Birnbaum M, Marchionni MA, Rutkowski JL (2001) Neuregulin signaling through a PI3k/Akt/bad pathway in Schwann cell survival. *Mol Cell Neurosci* 17:761–767.
30. Nave K-A, Salzer JL (2006) Axonal regulation of myelination by neuregulin 1. *Curr Opin Neurobiol* 16:492–500.
31. Rahmatullah M, *et al.* (1998) Synergistic regulation of Schwann cell proliferation by heregulin and forskolin. *Mol Cell Biol* 18:6245–6252.
32. Levi AD, *et al.* (1995) The influence of heregulins on human Schwann cell proliferation. *J Neurosci* 15:1329–1340.
33. Howe DG, McCarthy KD (2000) Retroviral inhibition of camp-dependent protein kinase inhibits myelination but not Schwann cell mitosis stimulated by interaction with neurons. *J Neurosci* 20:3513–3521.
34. Monje PV, Athauda G, Wood PM (2008) Protein kinase A-mediated gating of neuregulin-dependent ErbB2-ErbB3 activation underlies the synergistic action of camp on Schwann cell proliferation. *J Biol Chem* 283:34087–34100.
35. Nicholson SM, Gomes D, de Nechaud B, Bruzzone R (2001) Altered gene expression in Schwann cells of connexin32 knockout animals. *J Neurosci Res* 66:23–36.
36. Chandross KJ, Chanson M, Spray DC, Kessler JA (1995) Transforming growth factor-beta 1 and forskolin modulate gap junctional communication and cellular phenotype of cultured Schwann cells. *J Neurosci* 15:262–273.
37. Chanson M, Chandross KJ, Rook MB, Kessler JA, Spray DC (1993) Gating characteristics of a steeply voltage-dependent gap junction channel in rat Schwann cells. *J Gen Physiol* 102:925–946.

38. Neveu MJ, *et al.* (1994) Colocalized alterations in connexin32 and cytochrome P-450IIB1/2 by phenobarbital and related liver tumor promoters. *Cancer Res* 54:3145–3152.
39. Li Q, *et al.* (2007) Cytoplasmic accumulation of connexin32 protein enhances motility and metastatic ability of human hepatoma cells in vitro and in vivo. *Int J Cancer* 121:536–546.
40. Omori Y, *et al.* (2007) Pathological significance of intracytoplasmic connexin proteins: Implication in tumor progression. *J Membr Biol* 218:73–77.
41. Sato H, *et al.* (2007) Regulation of renal cell carcinoma cell proliferation, invasion and metastasis by connexin 32 gene. *J Membr Biol* 216:17–21.
42. Prost G, Bernier-Valentin F, Munari-Silem Y, Selmi-Ruby S, Rousset B (2008) Connexin-32 acts as a downregulator of growth of thyroid gland. *Am J Physiol Cell Physiol* 294:E291–E299.
43. Hirabayashi Y, *et al.* (2007) Membrane channel connexin 32 maintains Lin(-)/c-kit(+) hematopoietic progenitor cell compartment: analysis of the cell cycle. *J Membr Biol* 217:105–113.
44. Melanson-Drapeau L, *et al.* (2003) Oligodendrocyte progenitor enrichment in the connexin32 null-mutant mouse. *J Neurosci* 23:1759–1768.
45. Omori Y, Zaidan Dagli ML, Yamakage K, Yamasaki H (2001) Involvement of gap junctions in tumor suppression: Analysis of genetically-manipulated mice. *Mutat Res* 477:191–196.
46. Flachon V, Durand C, Selmi-Ruby S, Rousset B, Munari-Silem Y (2001) Connexin 32 fused to the green fluorescent protein retains its ability to control the proliferation of thyroid cells. *Cell Commun Adhes* 8:447–452.
47. Giepmans BN (2004) Gap junctions and connexin-interacting proteins. *Cardiovasc Res* 62:233–245.
48. Stout C, Goodenough DA, Paul DL (2004) Connexins: functions without junctions. *Curr Opin Cell Biol* 16:507–512.
49. Flores CE, Li X, Bennett MVL, Nagy JI, Pereda AE (2008) Interaction between connexin35 and zonula occludens-1 and its potential role in the regulation of electrical synapses. *Proc Natl Acad Sci USA* 105:12545–12550.
50. Orellana JA, *et al.* (2009) Modulation of brain hemichannels and gap junction channels by pro-inflammatory agents and their possible role in neurodegeneration. *Antiox Redox Signal* 11:369–399.
51. Abrams CK, Bennett MVL, Verselis VK, Bargiello TA (2002) Voltage opens unopposed gap junction hemichannels formed by a connexin 32 mutant associated with X-linked Charcot-Marie-Tooth Disease. *Proc Natl Acad Sci USA* 99:3980–3984.
52. Jessen KR, Mirsky R (1992) Schwann cells: early lineage, regulation of proliferation and control of myelin formation. *Curr Opin Neurobiol* 2:575–581.
53. Carroll SL, Miller ML, Frohnert PW, Kim SS, Corbett JA (1997) Expression of neuregulins and their putative receptors, ErbB2 and ErbB3, is induced during Wallerian degeneration. *J Neurosci* 17:1642–1659.
54. Hayworth CR, *et al.* (2006) Induction of neuregulin signaling in mouse Schwann cells in vivo mimics responses to denervation. *J Neurosci* 26:6873–6884.
55. Boutry JM, *et al.* (1992) Establishment and characterization of a mouse Schwann cell line which produces myelin in vivo. *J Neurosci Res* 32:15–36.